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New *Methyloceanibacter* diversity from North Sea sediments includes methanotroph containing solely the soluble methane monooxygenase

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Summary

Marine methylotrophs play a key role in the global carbon cycle by metabolizing reduced one-carbon compounds that are found in high concentrations in marine environments. Genome, physiology and diversity studies have been greatly facilitated by the numerous model organisms brought into culture. However, the availability of marine representatives remains poor. Here, we report the isolation of four novel species from North Sea sediment enrichments closely related to the Alphaproteobacterium *Methyloceanibacter caenitepidi*. Each of the newly isolated *Methyloceanibacter* species exhibited a clear genome sequence divergence which was reflected in physiological differences. Notably one strain R-67174 was capable of oxidizing methane as sole source of carbon and energy using solely a soluble methane monooxygenase and represents the first marine Alphaproteobacterial methanotroph brought into culture. Differences in maximum cell density of >1.5 orders of magnitude were observed. Furthermore, three strains were capable of producing nitrous oxide from nitrate. Together, these findings highlight the metabolic and physiologic variability within closely related *Methyloceanibacter* species and provide a new understanding of the physiological basis of marine methylotrophy.

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Introduction

Methylotrophs are key players in global marine carbon cycling (Redmond *et al.*, 2010; Chistoserdova, 2011; Halsey *et al.*, 2012; Beck *et al.*, 2013). They are capable of growth on methanol as well as other reduced methylated one-carbon (C_1) compounds such as methane, methylamine or formate (Hanson and Hanson, 1996; Trotsenko and Murrell, 2008; Chistoserdova, 2011). Methanol is one of the major oxygenated volatile organic compounds in marine systems with major sources being atmospheric deposition, phytoplankton excretion and the turnover of the potent greenhouse gas methane emitted from the seafloor and organic rich sediments by methane-oxidizing methylotrophs (further denoted as methanotrophs or MOB). Ocean surface concentrations of methanol can reach up to 100 nM in the Pacific Ocean (Singh, 2003) and 300 nM in the Northern hemisphere (Galbally and Kirstine, 2002), but it is unclear whether oceans are a methanol sink or source to the atmosphere (Williams, 1969; Singh, 2003; Heikes *et al.*, 2002; Carpenter *et al.*, 2004; Sinha *et al.*, 2007). Methylotrophic bacteria, together with mixotrophic eukaryotes, represent an oceanic sink for methanol, mainly through its use as energy source and to a lesser extent as carbon source (Dixon *et al.*, 2011). Using DNA-stable isotope probing *Methylophaga* spp. and other, novel *Gammaproteobacteria* were found as major consumers of methanol and methylamine, in surface seawater (Neufeld *et al.*, 2007). Cultivation-based studies have demonstrated that Alphaproteobacterial SAR 11, which are some of the most abundant heterotrophs in the oceans, use one-carbon compounds like methanol for energy generation (Sun *et al.*, 2011), while the Betaproteobacterial OM43 clade is a dominant marine methylotroph (Giovannoni

et al., 2008; Sowell *et al*., 2011) in productive coastal waters, including in the North Sea (Sekar *et al*., 2004).

Despite their crucial role in marine carbon cycling, only a handful of marine methanol oxidizers and even fewer methane oxidizers, the latter all belonging to the *Gammaproteobacteria*, have been isolated and physiologically characterized (Sieburth *et al*., 1987; Lidstrom, 1988; Fuse *et al*., 1998; Giovannoni *et al*., 2008; Boden *et al*., 2011; Huggett *et al*., 2012; Hirayama *et al*., 2013; Hirayama *et al*., 2014; Takeuchi *et al*., 2014a,b; Tavormina *et al*., 2015; Jimenez-Infante *et al*., 2016). Most methanotrophic surveys focused on high methane-loaded systems like mud vulcanoes (Niemann *et al*., 2006), vent fields (Nercessian *et al*., 2005) and methane gas seeps (Inagaki *et al*., 2004; Yan *et al*., 2006; Håvelsrud *et al*., 2011). These areas reveal a dominance of Gammaproteobacterial methanotrophs affiliated to the *Methylococcaceae* as the most abundant aerobic methanotrophs, with occasional molecular detection reports of Alphaproteobacterial methanotrophs (Inagaki *et al*., 2004; McDonald *et al*., 2005; Nercessian *et al*., 2005; Niemann *et al*., 2006; Valentine, 2011). Interestingly, pmoA amplicon sequencing demonstrated that more than 80% of detected sequences were attributed to novel species of as yet uncultured methanotrophs (Yan *et al*., 2006; Håvelsrud *et al*., 2011; Abdallah *et al*., 2014). As such, novel cultures remain pivotal to unravel complex biological traits important to marine biogeochemistry, especially since reports on high metabolic versatility among congenerics and even conspecifics (i.e. belonging to the same genus and species respectively) is accumulating (Vorobev *et al*., 2013; Beck *et al*., 2014; Hoefman *et al*., 2014). This metabolic versatility is a major confounding factor for linking biodiversity to ecosystem functions and further warrants the capture of a greater representation of marine methylotrophic diversity into pure culture (Hoefman *et al*., 2014). These cultures should serve to expand understanding of the traits that influence their activity patterns in marine systems such as the capacity to use alternative carbon and energy sources or the interaction with nitrogen and sulfur cycling (Klotz and Stein, 2008; Stein *et al*., 2011).

We now highlight the metabolic versatility with respect to methylotrophy and nitrogen metabolism among marine methylotrophs, discovered via shotgun sequencing of highly enriched methane-oxidizing cultures and subsequent isolation of the methylotrophs from these enrichments. Within the recently described methylotrophic genus *Methyloceanibacter*, we found representatives of four novel species. One of these new species is capable of methane oxidation, for which it uses solely the soluble methane monooxygenase (sMMO), making this the first Alphaproteobacterial methanotroph isolated from a marine system, the third methanotrophic taxon only possessing sMMO and the first methanotroph within a non-methanotrophic genus. Furthermore, converging with the species boundaries, *Methyloceanibacter* spp. displayed a wide physiological variation related to growth kinetics on methanol, and preferences for nitrogen, pH, temperature and salt.

Results

Enrichment and isolation of four novel Methyloceanibacter species

North Sea sediments from two sampling sites (W04, Vlakte van de Raan; W09, Hinderbanken) were incubated at 20°C with a headspace composition of 20:80 v/v methane/air under conditions mimicking the *in situ* nutrient availability. Methane oxidation was observed within one to two weeks, and after periodical headspace refreshments during two months, dense cultures were formed. Through extinction culturing under methane atmosphere, transfer of highest positive dilutions and numerous subcultivations over a period of three years, these two methane-oxidizing cultures were highly enriched. Subsequent metagenome sequencing revealed the presence of one highly enriched bacterium in sample W04 (representing 83.10% of the total reads) and two in sample W09 (together representing 87.04% of total reads). All three genomic bins were closely related with the recently described methylotrophic Alphaproteobacterium *Methyloceanibacter caenitepidi* Gela4^T, obtained from marine sediment collected near a hydrothermal vent in Japan (Takeuchi *et al*., 2014a). Surprisingly, no *pmoA* genes encoding the particulate methane monooxygenase (pMMO) were detected in the metagenomes. However, one genomic bin in each metagenome contained a complete operon encoding a sMMO, making it very likely that these *Methyloceanibacter* spp. were responsible for the observed methane oxidation of the enrichment cultures. Furthermore, the second bin in sample W09 seemed to be originating from a methylotrophic *Methyloceanibacter* sp. without sMMO encoding genes.

In a final effort to obtain pure cultures of *Methyloceanibacter*, methanol was used as carbon source and small white colonies were obtained on plate after two months. Forty-one *Methyloceanibacter* isolates (as verified by partial 16S rRNA gene sequencing) were picked up, genotyped with $(GTG)_5$ and Box rep-PCR fingerprinting and grouped into four separate clusters, all distinct from *M. caenitepidi* Gela4^T. One representative was chosen per cluster and designated with strain numbers R-67174, R-67175, R-67176, and R-67177 (Supporting Information Fig. S1). R-67174 was solely isolated from site W04, R-67175 and R-67177 solely from site W09, and R-67176 from both sites. Genomes of all four strains were sequenced (Supporting Information Table S1) and genomic taxonomy (Supporting Information Table S2) demonstrated that each strain represents a novel species

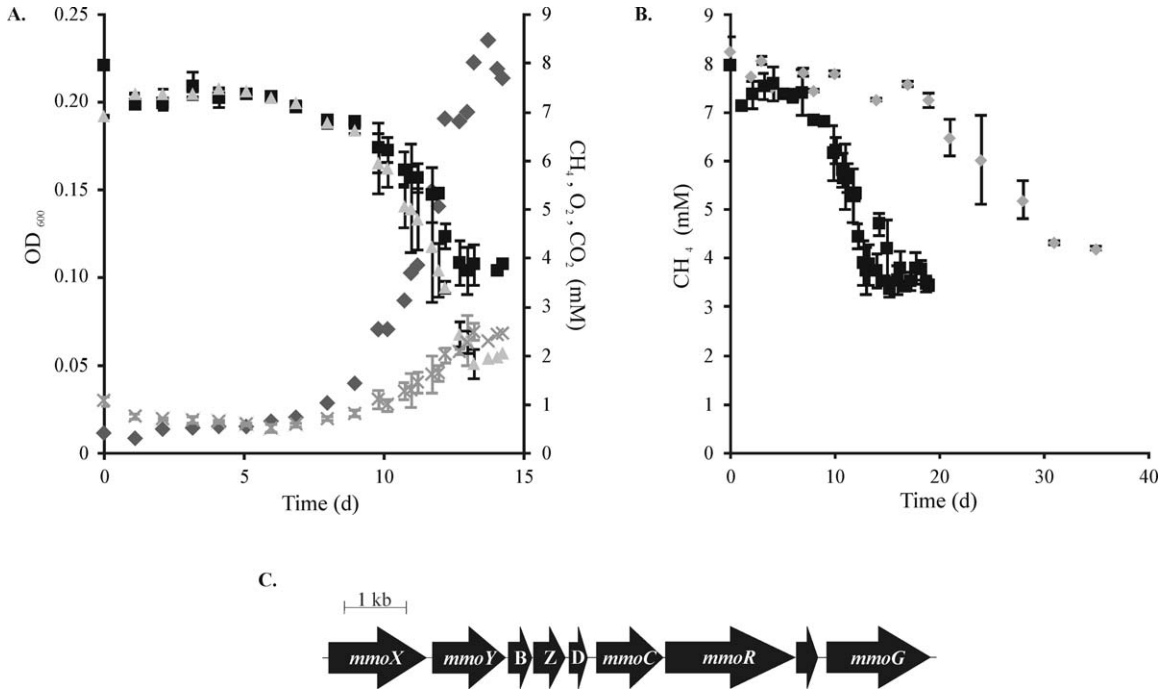

Fig. 1. Physiological (A, B) and genomic (C) evidence for methane oxidation by *Methyloceanibacter* sp. R-67174. (A) Growth under aerobic methane atmosphere amended with carbon dioxide was measured as OD_{600} (◆) ($n = 3$). It coincided with methane (■) and oxygen (▲) consumption and carbon dioxide (X) production, indicative of methane oxidation activity. (B) methane oxidation activity under aerobic methane atmosphere with (■) and without (◆) amendment with carbon dioxide ($n = 3$). Standard deviations are given as error bars but were omitted for OD_{600} for clarity. (C) A complete sMMO operon found in the genome supports the physiological observations. The open reading frames are drawn to scale and arrows show the direction of transcription.

within the genus *Methyloceanibacter* (Supporting Information Fig. S2). The strains clearly differed in growth morphology in liquid medium, with R-67174 forming small irregular flocs, R-67175 dense single cell cultures, R-67176 slightly dense single cell cultures with occasionally small flocs and R-67177 attaching firmly to the side of the vials. *M. caenitepidi* Gela4^T also formed single cell cultures, similar to R-67175. All strains formed identical colonies (round, white and convex) on solid media and displayed similar rod like shaped cell morphology, similar to *M. caenitepidi* Gela4^T, with Gram-negative staining (Takeuchi *et al.*, 2014a).

R-67174 showed a 100% 16S rRNA gene sequence similarity with both methanotrophic genomic bins of the W04 and W09 enrichments, although all isolates representing strain R-67174 originated from WE04. Its methane oxidation capacity was indeed confirmed physiologically and its genome contained a complete operon encoding a sMMO (see below).

C1 metabolism

Use of methane and methanol as sole source of carbon and energy was tested for all *Methyloceanibacter* strains including *M. caenitepidi* Gela4^T. Only R-67174 demonstrated growth coupled to methane and oxygen decrease and carbon dioxide increase over time (Fig. 1A). The addition of extra carbon dioxide to the headspace had a growth-promoting effect by decreasing the lag phase, as could be seen from the methane oxidation profile (Fig. 1b). This effect was not linked to an acidification of the media as no significant pH shift was measured under increased carbon dioxide concentrations (data not shown). Furthermore cultures grown to stationary phase reached similar maximal cell densities (appr. 23×10^7 cells/mL) refuting autotrophic growth, which is supported by the lack of RubisCO genes necessary for fixation of the inorganic carbon dioxide into organic carbon. Thus, as R-67174 is a Alphaproteobacterial methanotroph, the effect of carbon dioxide is most likely linked to use of the serine pathway for carbon assimilation (Supporting Information Table S3), which requires one carbon dioxide for each formaldehyde (Chistoserdova, 2011). As was expected from the metagenome data, the genome of R-67174 lacked *pmo* genes and contained a complete *mmoXYBZDC* operon for a sMMO (Fig. 1c). MmoX was most affiliated with that of *Methylocella silvestris* (Dunfield *et al.*, 2003) and *Methyloferulla stellate* (Vorobev *et al.*, 2011) (92.6 and 91.3% aa similarity) (Fig. 2), both Alphaproteobacterial methanotrophs with solely a sMMO. The genome environments of

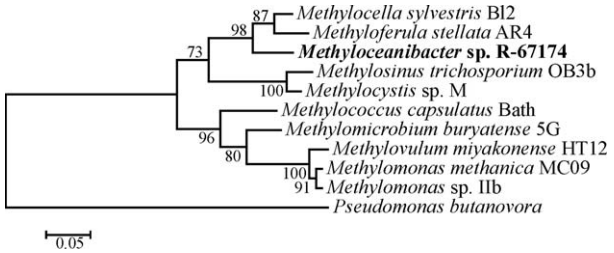

Fig. 2. Phylogenetic Maximum Likelihood tree showing the affiliation of the soluble methane monooxygenase. The evolutionary history was inferred by using the Maximum Likelihood method based on the Le_Gascuel_2008 model (Le and Gascuel, 2008) using 473 amino acid position. The tree with the highest log likelihood (−3557.4323) is shown. Bootstrap values (1000 replicates) higher than 70 are shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.5761)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Alignment and evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). Only complete sequences from genomes were included. Butane monooxygenase of *Pseudomonas butanovora* was used as outgroup.

their sMMO operons were identical and mainly differed from other methanotrophs in the localization of adjacent *mmoR* and *mmoG* genes (Supporting Information Fig. S3).

R-67174 clearly demonstrated preferential growth on methanol compared to methane (Table 1), which is a common observation for pMMO lacking methanotrophs (Dunfield *et al.*, 2003; Vorobev *et al.*, 2011), with a comparable effect of carbon dioxide addition as mentioned higher for methane (shortened lag phase without increased maximum cell density or specific growth rate). Also when both carbon sources were given at the same time, R-67174 preferred methanol as carbon and energy source: methane concentrations remained unaltered over time as growth progressed (Supporting Information Fig. S4), while lag phase, specific growth rate and maximal cell density did not differ significantly from those on methanol alone (Table 1). A qualitative naphthalene oxidation assay on mid-exponentially grown methanol-fed R-67174 cells indicated the sMMO was not expressed (data not shown). This was confirmed by a quantitative enzyme activity test where no methane oxidation was detected in methanol fed R-67174 cells or heat killed methane fed R-67174 cells over time compared to an methane oxidation activity of 0.024 mM CH_4/h (±0.012) in mid exponentially grown methane fed R-67174 cells.

All strains demonstrated growth with methanol as the sole carbon and energy source (Fig. 3). Strikingly, statistically significant strain-specific differences in kinetic parameters were observed (Fig. 3, Table 1). Gela4^T had the shortest lag phase and highest growth rate, while R-67175 needed more than eight days to start exponential growth but then reached the highest maximum cell density. In comparison, growth of R-67177 was more than five time slower and reached stationary phase at a cell density almost 1.5 order of magnitude lower. All the genomes contained a single gene cluster (*mxaFJGIRSACKLD*) encoding the canonical MxaFI methanol dehydrogenase (Supporting Information Table S3). MxaF sequences of *Methyloceanibacter* spp. were highly similar (from 100% between Gela4^T and R-67175 to 84.8% aa similarity between R-67174 and R-67177) and were most affiliated with MxaF from *Methyloligella solikamskensis* (Supporting Information Fig. S5) (78.8 - 89.4% aa similarity). Other essential genes for the expression of the pyrroloquinoline quinone catalytic cofactor (*pqq*ABCDE) were found (Supporting Information Table S3). The genomes also contained multiple *xoxF* genes (Pol *et al.*, 2014; Keltjens *et al.*, 2014; Taubert *et al.*, 2015), coding for the lanthanide dependent methanol dehydrogenase (Supporting Information Fig. S5; Supporting Information Table S3). Gela4^T, R-67174 and R-67176 had four *xoxF1* copies (two adjacent and two separated), R-67175 two adjacent *xoxF1*, and R-

Table 1. Kinetic parameters for growth on 1% methanol for different *Methyloceanibacter* spp.

Strain	Condition	Lag phase λ (d)	Specific growth rate (h^{-1})	Generation time (h)	Maximum cell density (10^8 cells mL^{-1})
R-67174	Methane + carbon dioxide	8.96^{a} (0.43)	0.017^{a} (0.001)	41.85^{a} (2.49)	2.32^{a} (0.06)
	Methanol	2.97^{bA} (0.25)	0.033^{bA} (0.002)	21.07^{bA} (1.44)	4.19^{bA} (0.14)
	Methanol + carbon dioxide	2.01^{c} (0.26)	0.033^{b} (0.002)	20.98^{b} (1.52)	4.62^{b} (0.27)
	Methane + methanol + carbon dioxide	1.76^{c} (0.34)	0.032^{b} (0.001)	21.40^{b} (0.94)	4.28^{b} (0.32)
R-67175	Methanol	8.23^{B} (0.15)	0.021^{B} (0.002)	33.06^{B} (2.63)	47.43^{B} (0.79)
R-67176	Methanol	2.72^{A} (0.14)	0.049^{C} (0.005)	14.14^{C} (1.44)	14.48^{C} (0.31)
R-67177	Methanol	6.11^{C} (0.59)	0.012^{D} (0.001)	57.67^{D} (2.43)	1.22^{D} (0.06)
Gela4^T	Methanol	1.57^{D} (0.09)	0.069^{E} (0.002)	10.02^{E} (0.31)	35.00^{E} (0.69)

Standard deviations are given between brackets (based on at least three replicate measurements). Significance ($p < 0.01$) of between-strain differences for growth on methanol and within-strain differences per growth condition for R-67174 were determined via one-way ANOVA (see Materials and Methods for details) and are displayed as different upper- and lowercase letters, respectively (combined letters are used to indicate non-significance for multiple variables).

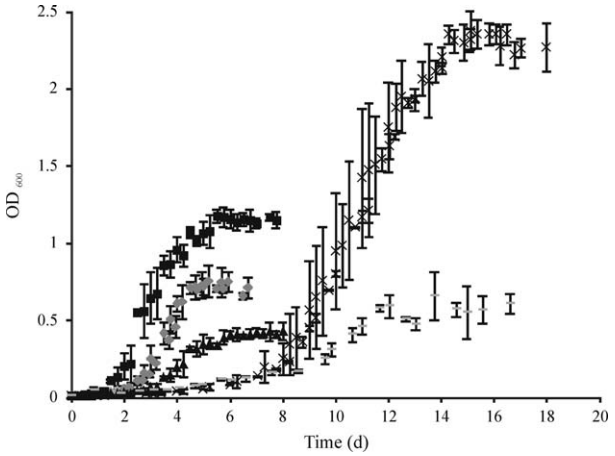

Fig. 3. Growth on 1% methanol (v/v) for different *Methyloceanibacter* spp over time. R-67174 (▲), R-67175 (X), R-67176 (◆), R-67177 (−), Gela4T (■). All strains were grown till stationary phase (based on at least 3 replicate measurements) and demonstrated clear differences in lag phase, growth rate and maximum cell density (see Table 1).

67177 two *xoxF1* copies (and two truncated genes located separately) as well as one complete *xoxF3*. All XoxF1 grouped into three cluster (Supporting Information Fig. S5; clusters C1, C2, C3), with >90% aa similarities within each cluster. R-67174, R-67175 and R-67177 also have the genomic potential to encode a PQQ-ADH type 9 quinoproteins (Fig. S5) (Keltjens *et al.*, 2014). Unfortunately, there is no clear link between the *xoxF* gene inventories and the kinetic parameters on methanol. But *xoxF* expression in methylotrophs containing calcium-dependent MxaF has already been shown in the marine environment for *Methylophaga* (Grob *et al.*, 2015). So, considering that lanthanides (specifically cerium, lanthanum, praseodymium and neodymium) were detected in the 0.1 to 10 nanomolar range in pore water of the sediment at each sampling site in this study and excess of these lanthanides were always added to the growth medium (throughout the enrichment procedure and during growth experiments), it is plausible that their differential expression might have contributed to the observed significant strain-dependent differences in growth on methanol.

Differential nitrogen preferences, alternative carbon source utilization and responses to environmental stimuli

Detailed physiological and genomic data revealed clear differences in the nitrogen metabolism between the *Methyloceanibacter* strains. All strains have the genomic potential to transport and assimilate ammonium via glutamine synthetase – glutamate synthase (GS-GOGAT) (Supporting Information Table S3). While the GS-GOGAT would operate under low ammonium and nitrate concentrations, glutamate dehydrogenase, present in R-67177, and to a lesser extent alanine dehydrogenase, present in R-67175, could be used when ammonium concentrations are high (Supporting Information Table S3) (Lees *et al.*, 1991). Further the presence of other enzymes capable of direct ammonium assimilation, i.e. NAD synthethase, carbamoyl phosphate synthetase and asparagine synthetase, in all strains might further support the assimilation of ammonium (van Heeswijk *et al.*, 2013). Next strains Gela4^T, R-67175 and R-67177 encoded enzymes for the transport and assimilatory reduction of nitrate (Supporting Information Table S3). All strains indeed utilized ammonium as sole nitrogen source within a range of 0.5 to 10 mM (Fig. 4; Supporting Information Table S4), with mostly broad concentration range between 2 and 10 mM but with a well-defined preference at 10 mM for R-67176. For nitrate as sole nitrogen source, R-67175 and R-67177 showed highest cell density at 5 mM and Gela4^T at 10 mM (Fig. 4; Supporting Information Table S4). Genes for the dissimilatory membrane-bound cytoplasmic nitrate reductase NarG and one or two NarK-type nitrate/nitrite antiporters were found in all genomes except that of the methanotroph R-67174 (Supporting Information Table S3). NarG aa similarity ranged between 82.8-99.8% and its phylogeny agreed with that of the 16S rRNA gene (Fig. 2) and MxaF (Supporting Information Fig. S5), suggesting that R-67174 might have lost the *nar* operon quite recently as it still contained a *narK*. Although dissimilatory nitrate reduction was not experimentally verified, nitrous oxide production by Gela4^T, R-67175 and R-67177 grown on nitrate was observed at end exponential/early stationary phase (at oxygen concentration between 100-300 μM), suggesting that dissimilatory nitrate reduction proceeds once oxygen is depleted. The amount of produced nitrous oxide appeared correlated with the initial nitrate concentration and 4 to 8% of initial nitrate was converted to nitrous oxide (data not shown). This low conversion rate combined with the absence of denitrification genes in the genomes, the presence of several genes for nitrosative stress response regulators (NsrR, NnrR, NnrS and NnrU) and nitrous oxide production being restricted to strains able to assimilate nitrate led us to hypothesize that nitrous oxide is produced via a nitrite detoxification pathway. Similar to nitrate-reducing *Enterobacteriaceae* (Smith, 1983; Corker and Poole, 2003; Gilberthorpe and Poole, 2008), high - potentially toxic - levels of produced nitrite could be converted to nitric oxide via the dual action of Nar and/or NirB. However, genes encoding the usual suspects for the further reduction to nitrous oxide, namely dedicated nitric oxide reductases like qNorB and Cu_ANor or flavohemoglobin Hmp were absent from the genomes. Lastly, the genomes of R-67174 and R-67176 also contained a complete gene inventory for nitrogen fixation (Supporting Information Table S3), with gene organization similar to that of *Methylocella silvestris* BL2 and a NifH aa sequence similarity of

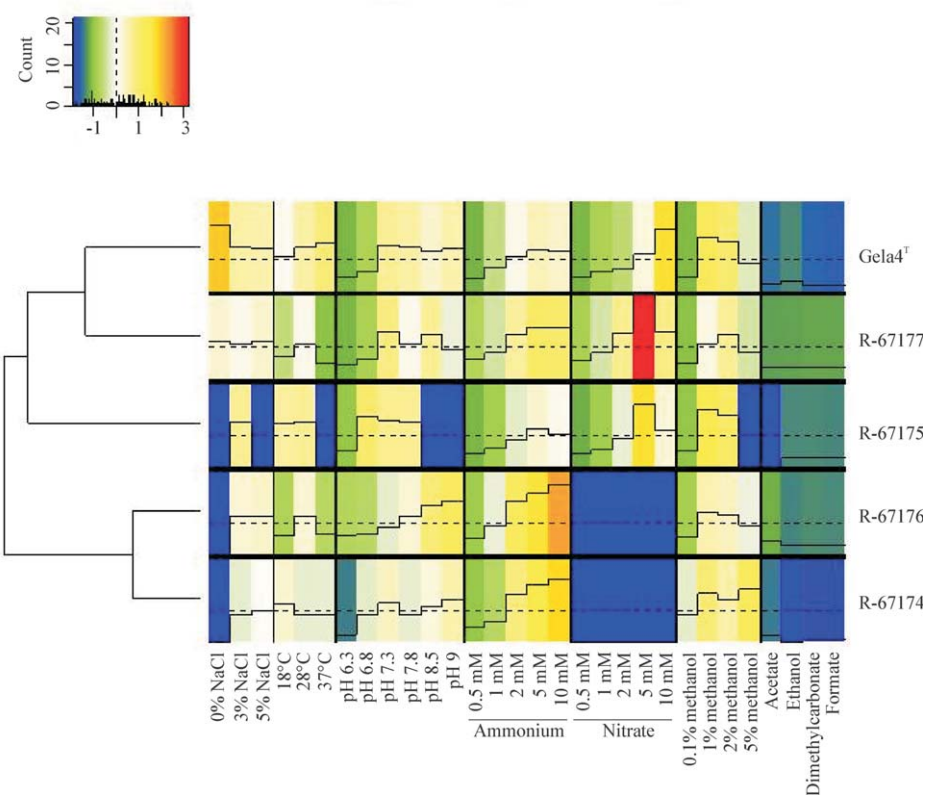

Fig. 4. Heat map of differential growth responses (OD_{600}) of the *Methyloceanibacter* spp (see Table S4). Colors and full lines represent normalized OD_{600}values. Values on the x-axis represent normalized OD_{600} values. These values are represented by the colors, where blue indicates no growth and red indicates highest growth. Counts on the y-axis represent the amount of times a certain value was reported. Rows of the matrix are clustered using complete hierarchical clustering.

95.9%. Although it was not experimentally verified under low oxygen tension, nitrogen fixation could compensate for the inability to assimilate nitrate under ammonium limitation or provide a competitive benefit in absence of (in)organic nitrogen sources.

Significant strain dependency of growth kinetics on methanol were already described above (Table 1), but, similar to the observed nitrogen preferences, differences became apparent for the different strains in relation to methanol concentrations, alternative carbon source utilization and responses to environmental stimuli (Fig. 4; Supporting Information Table S4). All strains except R-67175 grew with 0.1 to 5% methanol. R-67174 displayed a similar growth over this concentration range, while the others clearly preferred 1–2%. They were also all facultative methylotrophs able to use dimethylcarbonate and formate. In addition, only R-67175 could not utilize acetate, while only R-67174 was unable to grow on ethanol. However, maximal cell densities on these alternative carbon and energy sources were low compared to methanol, except for Gela4^T on ethanol and R-67176 on acetate. Both compounds are realistic alternative carbon sources *in situ* reaching up to 2–33 nM for ethanol (Beale *et al.*, 2010) and from 1 to 10 μM for acetate (King, 1991; Wellsbury, 1995; Wu *et al.*, 1997) in marine sediments. R-67177 seemed to have adapted to a lifestyle of a generalist, without a clear preference for either carbon source. Next to carbon metabolism, variation in growth ranges for salinity, temperature and pH further underlined strain-dependent physiologies of the *Methyloceanibacter* strains (Fig. 4, Supporting Information Table S4). R-67177 and Gela4^T demonstrated the broadest salinity range supporting growth, with no differences in maximum cell density for the former while the latter had an unexpected optimum at 0% salinity (given *in situ* salinity of 33-35 psu). In contrast, R-67174 and R-67176 needed salt to grow, and R-67175 was limited to a salinity of 3%. Also the temperature range of R-67175 (18–28°C) was narrower than that of the others (18–37°C). Again it was strain-dependent whether maximal cell densities varied with temperature. A similar observation was made for pH, with R-67175 being restricted to pH 6.3 to 7.8.

Discussion

In this study we were able to isolate a set of four marine methylotrophic *Methyloceanibacter* strains, in addition to the earlier described *M. caenitepidi*, that were categorized into five different species based on their ecophysiological variation and the divergence of their genomic sequences, of which four were novel. Surprisingly, one strain (represented by R-67174) was capable of methane oxidation, making this the first report on a methanotroph within a methylotrophic genus. Some claims were made in the past

(Patt *et al*., 1974; 1976; Van Aken *et al*., 2004), but could never be independently verified (Dedysh *et al*., 2004a). Furthermore, its sole use of a sMMO and its marine origin as Alphaproteobacterial methanotroph makes strain R-67174 a unique discovery. *Methyloceanibacter* is the first genus with pMMO lacking methanotrophs retrieved from marine systems and only the third overall, next to *Methylocella* and *Methyloferrula* (Dedysh *et al*., 2000; Vorobev *et al*., 2011), also phylogenetically the most closely related methanotrophs (Fig. 2; Supporting Information Fig. S2). Given the scattered phylogenetic distribution of the sMMOs among methanotrophs (Leahy *et al*., 2003), its soluble nature requiring fewer genes than the particulate form and its promiscuity (Jiang *et al*., 2010), it is very plausible that horizontal gene transfer (HGT) is responsible for the spread of the trait. Trait acquisition should enable recipients to colonize a wider range of habitats than possible with the pMMO alone (Leahy *et al*., 2003) or without any MMO. Nevertheless, to date no hard evidence or experimental data for HGT of sMMO has been reported. We found a deviation in GC content of *mmoXYBZCRG* (56%) compared to the average GC content of the genome (64%) in R-67174, which might point to a recent acquisition, but the gene cluster was completely flanked by hypothetical proteins and its immediate genome environment lacked actual remaining traces of HGT. As the sMMO machinery is the sole distinctive factor separating methanotrophs from other methylotrophs, the occurrence of methanotrophs within strict methylotrophic clades is most likely widespread but problematic to observe. Liquid extinction cultures under methane headspace, as in this study, might consist of a population of genotypically distinct congenerics or conspecifics not easily differentiated at morphological, physiological or genomic levels. Once transferred to solid media under methane headspace, the non-methane oxidizing methylotrophs are lost. All four pMMO lacking methanotrophs (*Methylocella sylvestris, M. tundrae, M. palustris and Methyloferulla stellata*) belong to the *Alphaproteobacteria*, with R-67174 being the first isolated from a marine environment. Its isolation is not trivial as currently only eight marine methanotrophs are available in culture, *Methylomicrobium pelagicum* (Sieburth *et al*., 1987), *Methylobacter marinus* (Lidstrom, 1988), *Methylomicrobium japanense* (Fuse *et al*., 1998), *Methylomonas methanica* (Boden *et al*., 2011), *Methylomarinum vadi* (Hirayama *et al*., 2013), *Methylocaldum marinum* (Takeuchi *et al*., 2014b), *Methyloprofundis sedimenti* (Tavormina *et al*., 2015) and *Methylomarinovum caldicuralii* (Hirayama *et al*., 2014), all of which are Gammaproteobacterial MOB. Only three of them contain sMMO in addition to the pMMO (Fuse *et al*., 1998; Boden *et al*., 2011; Takeuchi *et al*., 2014b). The availability of marine methanotrophs *ex situ* clearly reflects the *in situ* situation, where Alphaproteobacterial MOBs have a widespread environmental distribution (Rockne and Strand, 2003; Rahman *et al*., 2011) but Gammaproteobacterial MOBs are numerically dominant in marine systems.

Although very closely related, genomic analysis, supported by ecophysiological analysis suggests the five *Methyloceanibacter* strains might occupy distinct ecological niches and/or potentially adhere to different life strategies, as evidenced by their differential kinetics of the methanol metabolism, nitrogen preferences, alternative carbon source utilization and optima for temperature, salt and pH. On example is the potential of R-67174 to use methane as sole source of carbon and energy. Acquisition of the methane oxidation capacity might give R-67174 a competitive advantage under methanol limitation. Like *Methylocella* and *Methyloferrula* (Dedysh *et al*., 2004b; Vorobev *et al*., 2011), it also preferred growth on methanol over methane (evidenced by higher growth rate and shorter lag phase), which now seems characteristic for pMMO-lacking methanotrophs. Therefore, R-67174 might behave as a methylotroph *in situ*, as stable isotope probing already revealed *Methylocella* spp. cross-feeding on the excreted products of other methanotrophs rather than oxidizing methane as a primary carbon source (C. Murrell, personal communication). In addition, methane oxidation can bestow R-67174 with the benefit of receiving secondary metabolites like vitamins or growth promoting factors from co-existing microorganisms in return for 'leaking' methane-derived carbon compounds (Iguchi *et al*., 2011). Another example concerns the capacity to fixate nitrogen or assimilate nitrate. Both processes were found to be mutually exclusive in the *Methyloceanibacter* strains and would therefore ensure assimilation of alternative nitrogen sources under ammonium limitation. Based on our observations, indicating the divergence of their genomic sequences and their ecophysiology, the *Methyloceanibacter* strains can be categorized into five different ecotypes (Fig. 4). Wide ecotype variation among methylotrophs seems widespread. For example ecotypes have been identified among *Methylophilaceae* isolated from Lake Washington; these were characterized by differential responses to specific C1 stimuli (Beck *et al*., 2014), environmental adaptation strategies (Vorobev *et al*., 2013) and nitrate removal potential (assimilatory versus respiratory nitrate reduction) (Beck *et al*., 2014). Noteworthy is that ecological population boundaries frequently occur at deep phylogenetic levels (Johnson *et al*., 2006; Hunt *et al*., 2008; Pittera *et al*., 2014; Salter *et al*., 2015), which might preclude meta-omics approaches from uncovering essential trait differences among closely related strains with identical biomarker sequences. This deep-phylogenetic trait separation has already been demonstrated for the nitrogen metabolism of terrestrial methanotrophic *Methylomonas* strains (Hoefman *et al*., 2014). The organisms obtained in this study provide novel experimental models

for studying the complexity and function of the microbial community active in methylotrophy.

The physiological and genomic data presented here helped to define strain variation within methylotrophic bacteria and to disentangle the phylogenetic levels at which environmental variables influence marine methanotrophy and methylotrophy. Besides carbon metabolism, nitrogen cycling is confirmed to play a fundamental role in shaping methylotrophic populations and salinity preferences will determine the localization of specific *Methyloceanibacter* strains in more coastal areas. The diverse metabolic features demonstrated here among only a small set of closely related methylotrophs, relative to the great genetic diversity revealed by ongoing metagenomic surveys, underlines the need for ongoing efforts in obtaining marine representatives *ex situ*. A change in isolation strategy targeting marine methanotrophs to broad screening of methanol-grown cultures will also aid to find more pMMO-lacking methanotrophs.

Description of *Methyloceanibacter methanicus* sp. nov.

Type strain: R-67174, LMG 29429

Etymology. N. L. neut. n. *methanum*, methane; L. masc. suff. *-icus*, adjective forming suffix used with the sense of pertaining to; N.L. masc. adj. *methanicus*, related to or associated with methane

Locality. Isolated from marine sediment in the North Sea at the station 'Vlakte van de Raan' (N51.449166 E3.237166)

Properties. Gram-negative, non-motile rods, liquid cultures form small microcolonies. Cells grow optimally at 18-37°C and pH 6.3-9 but requires NaCl at 3-5%. Grows aerobically on methane using a soluble methane monooxygenase but prefers methanol. Grows better with carbon dioxide added to headspace. Also grows using alternative carbon sources acetate, formate and dimethylcarbonate. Requires ammonium as inorganic nitrogen source. The G + C content of the type strain is 64.0 mol%.

Description of *Methyloceanibacter superfactus* sp. nov.

Type strain: R-67175, LMG 29430

Etymology. L. prep. *super*, above, on top; N.L. v. *facere*, to produce by growth; *superfactus*, referring to high maximal cell densities.

Locality. Isolated from marine sediment in the North Sea at the station Hinderbanken (N51.75 E2.7)

Properties. Gram-negative, non-motile rods. Cells grow optimally at 18–28°C and pH 6.3–7.8 but requires 3% NaCl. Grows aerobically on methanol. Also grows using alternative carbon sources ethanol, formate and dimethylcarbonate. Capable of using ammonium and nitrate as inorganic nitrogen sources. The G + C content of the type strain is 64.5 mol%.

Description of *Methyloceanibacter stevinii* sp. nov.

Type strain: R-67176, LMG 29431

Etymology. *stevinii*, of Stevin, referred to the Belgian scientific research vessel 'Simon Stevin', used to collect the samples at the North Sea.

Locality. Isolated from marine sediment in the North Sea at the station Vlakte van de Raan (N51.449166 E3.237166) and at the station Hinderbanken (N51.75 E2.7)

Properties. Gram-negative, non-motile rods, liquid cultures are single cells with occasional flock formation. Cells grow at 18–37°C and pH 6.3–9 but requires NaCl at 3–5%. Grows aerobically on methanol. Also grows using alternative carbon sources ethanol, acetate, formate and dimethylcarbonate. Requires ammonium as inorganic nitrogen source. The G + C content of the type strain is 63.1 mol%.

Description of *Methyloceanibacter marginalis* sp. nov.

Type strain: R-67177, LMG 29432

Etymology. L. n. *margo*, edge, margin; L. masc. suff. *-alis*, suffix denoting pertaining to; N.L. masc. adj. *marginalis*, referring to cells stick to the edge of the recipient in liquid culture.

Locality. Isolated from marine sediment in the North Sea at the station Hinderbanken (N51.75 E2.7)

Properties. Gram-negative, non-motile rods, liquid cultures grow attached to the side of the recipient and form flocks. Cells grow at 18–37°C, pH 6.3–9 and 0–5% NaCl. Grows aerobically on methanol. Also grows using alternative carbon sources ethanol, acetate, formate and dimethylcarbonate. Capable of using ammonium and nitrate as inorganic nitrogen sources. The G + C content of the type strain is 63.5 mol%.

Experimental procedures

Sampling, enrichment and isolation

Marine surface sediments were collected in 2012 at two different stations in the Belgian North Sea, W04 (Vlakte van de Raan, N 51.449166 E3.237166) and W09 (Hinderbanken, N 51.75 E2.7), and used as inocula for enrichments. Initial enrichments were performed using media mimicking the *in-situ* nutrient conditions, based on *in-situ* concentrations measured periodically by the Belgian Marine Datacenter (http://www.mumm.ac.be/datacentre/) over the last 24 years. Phosphate concentrations were adjusted to the Redfield ratio (N/P: 16/1) (Cleveland and Liptzin, 2007) as data for phosphate was unavailable. Media composition was as follows: 25 μM KNO_3, 0.5 μM $NaNO_2$, 10 μM NH_4Cl, 4 μM KH_2PO_4, 1 μM $CuSO_4.5H_2O$, 30 μM FeNaEDTA for W04; 5 μM KNO_3, 0.3 μM $NaNO_2$, 3 μM NH_4Cl, 1.5 μM KH_2PO_4, 1 μM $CuSO_4.5H_2O$, 10 μM FeNaEDTA for W09. All media were prepared with aged seawater, collected at three meter depth

from the sampling point. Enrichments were incubated at 20°C, shaking at 100 rpm and a headspace composition of 20:80 v/v methane/air.

After methane oxidation was observed and dense culture developed, the enrichments underwent an extinction culturing in NaCl-dANMS media (Holmes *et al.*, 1995) supplemented with 1 mM KH_2PO_4, 500 μM NH_4Cl, 500 μM KNO_3, 1 μM $CuSO_4.5H_2O$, 40 μM FeNaEDTA, 100 nM of the lanthanides $LaCl_3$, $CeCl_3.7H_2O$, $NdCl_3.6H_2O$, $PrCl_3$ and 5 mM HEPES at a final pH of 7.8. Highest dilutions showing growth were transferred and underwent numerous subcultivations over a period of three years. Repeated subcultivations were not sufficient to purify the methane-oxidizing bacteria but resulted in a stable MOB-heterotroph community. Isolation of the strains was performed by plating on the growth medium solidified with Gelzan (1% w/v) and supplemented with 0.5% methanol. After several months of incubation small white colonies formed on the highest dilution plates. These were picked and placed in 5 mL liquid medium containing 0.5% methanol as sole carbon source. Picked colonies were identified using partial 16S rRNA gene sequences, as described previously (Heylen *et al.*, 2006), and only isolates affiliated with *Methyloceanibacter* were retained. Purity was checked routinely through (i) microscopic evaluation and (ii) plating on 1/10 Trypticase Soy agar (TSA) under air atmosphere.

Genotyping and next generation sequencing

Genomic DNA was obtained by using Guanidium-thiocyanate-EDTA-sarkosyl for the enrichment cultures (Pitcher *et al.*, 1989) or hexadecyltrimethylammonium bromide (CTAB) for the pure cultures (Cleenwerck *et al.*, 2009). DNA quality and quantity were checked on agarose gel and with Qubit Fluorometric Quantitation.

Pure cultures were typed by performing $(GTG)_5$ and Box rep-PCR fingerprinting as previously described (Heyrman, 2005). Random shotgun sequencing of pure and enrichment cultures were done using the IonTorrent PGM as described previously (Russ *et al.*, 2014). Analysis of reads was performed using SPAdes genome assembler v.3.5.0 (Bankevich *et al.*, 2012) and CLC genomics workbench v7.0.4 (CLCbio, Denmark). The reads were length and quality score trimmed and used for *de novo* assembly. Contig binning of metagenomes from enrichment cultures was done by comparing GC content to sequencing depth and resulted in one and two draft genomes for enrichment W04 and W09 respectively. The Rapid Annotation Subsystem Technology (RAST) server was used for functional annotation and metabolic reconstruction of the (meta)genomes (Aziz *et al.*, 2008; Overbeek *et al.*, 2014). The classic RAST annotation scheme was selected using RAST gene caller which allowed automatic error fixing, frameshift correction and the backfilling of gaps. Assigned functions were verified with PSI-BLAST (Altschul, 1997). Missing genes were searched for in the genome with pBLAST using homologous amino acid sequences from the closely related organisms.

Ecophysiology

Growth of the different strains in different culture conditions was assessed in batch cultures (5 mL) by varying carbon source, nitrogen source, temperature, salinity and pH. All strains were grown at 28°C in NaCl-dANMS media (Holmes *et al.*, 1995), i.e. dANMS supplemented with 3% NaCl, containing a concentration of 1 μM $CuSO_4.5H_2O$, 1 mM KH_2PO_4, 2 mM KNO_3, 2 mM NH_4Cl, 40 μM FeNaEDTA, 100 nM of the lanthanides $LaCl_3$, $CeCl_3.7H_2O$, $NdCl_3.6H_2O$, $PrCl_3$ and 5 mM HEPES, unless indicated otherwise. Methanol (1% v/v) was used as the sole carbon and energy source. Each growth conditions was tested in triplicate. Utilization of the following alternative carbon sources (0.03%) was evaluated by monitoring growth in media without methanol: tryptic soy broth, acetate, acetate, formate, methylamine, dimethyl sulfoxide, dimethylcarbonate, formamide, ethanol, and no carbon source as negative control. Utilization of nitrate, ammonium was tested at concentrations of 0.5 mM, 1 mM, 2 mM, 5 mM and 10 mM. Alternatively yeast extract (0.03% w/v) was added to test as nitrogen source. The ability to fix nitrogen was assessed by evaluating growth in nitrogen free medium with 1% methanol under atmospheric concentrations of oxygen (20.94% O_2). Nitrous oxide production was tested for the different nitrate and ammonium concentrations by measuring headspace composition after 6 weeks growth. Salt tolerance (tested at 0%, 3%, 5% and 10% NaCl w/v spiked), pH range (tested at 5.8, 6.3, 6.8, 7.3, 7.8, 8.5, 9, 10 and 11) and temperature range (10°C, 18°C, 28°C, 37°C, 45°C and 52°C) were also determined.

Growth kinetics on methane and methanol

Cells harvested from mid-exponentially grown cultures were used as inoculum to start methane and methanol growth curves. Cells were washed twice with fresh carbon free medium and inoculated to a final starting OD_{600} of 0.01. Methane oxidation was tested in 15 mL serum vials, containing 2.5 mL dANMS medium supplemented with 3% NaCl and 1% methanol. Based on initial tests, methane oxidation by strain R-67174 was clearly inhibited partially or completely by black non halogenated butyl rubber stoppers (Niemann *et al.*, 2015). As such, vials were capped with grey halogenated Chlorobutyl stoppers (VWR® Chlorobutyl Serum and Lyophilization Stoppers) and incubated at 28°C, by shaking at 100 rpm and a headspace composition of either 20:80 v/v methane/air, 5:20:75 carbon dioxide/methane/air or 5:95 carbon dioxide/air. Per time point headspace composition analysis and biomass determination of three serum vials were analyzed, which were subsequently discarded from the experiment. Because of attached cell growth to the serum vials glass by strain R-67177, cell flocks were disrupted with a manual glass homogenizer before biomass determination.

Carbon source preference and sMMO activity assay

Preferential use of methane or methanol as carbon source was tested using mid-exponentially grown cells as inoculum at a starting OD_{600} of 0.01 in 200 mL dANMS medium in 1L bottles. Medium was spiked with methanol (v/v) to a final concentration of 1% under a headspace composition of 5:20:75 carbon dioxide/methane/air. Subsequently growth and methane were monitored over time in all six replicates. As negative control sterile medium was used and incubated under the same conditions. Qualitative sMMO expression was

tested by the naphthalene oxidation assay in liquid cultures (Brusseau *et al.*, 1990). Napthol inoculated cultures and methane grown cultures were used as positive control. Quantitative sMMO activity assay was performed as described previously (Nyerges and Stein, 2009). In short, 10^9 cells of mid-exponentially grown methanol fed cultures were harvested and washed twice in HEPES buffer (10 mM, pH 7.8), suspended in 1 mL HEPES buffer and placed into 60 mL glass vials sealed with grey butyl rubber stoppers and aluminum crimp seals. Methane and carbon dioxide were added to a final concentration of 5:20:75 carbon dioxide/methane/air, vials were incubated shaken (100 rpm) at 28°C and the headspace was sampled every hour during 5h and after 24h. Negative controls consisted of heat killed cells incubated under identical conditions. Three replicates were assayed per sample per time point.

Analytical methods

Growth was monitored over time by optical density at 600 nm using a Spectramax plus 384 spectrophotometer (Molecular devices). Mid-exponentially grown cells were used to correlate biomass to cell counts using live/dead flow cytometry as described previously (Van Nevel *et al.*, 2013). Concentrations of CH_4, O_2 and carbon dioxide were measured with a Compact GC (Global Analyzer Solutions, Belgium), equipped with one channel connected to a flame ionization detector and two channels connected to a thermal conductivity detector. Values were converted to μmol/L for methane, oxygen and carbon dioxide by compensating for change in gas pressure (measured with an Infield 7 pressure meter, UMS, Germany) and taking the solubility of the gases into account. Methanol was analyzed using a 930 Compact IC Flex (Metrohm, Switzerland) ion chromatography system. Separation occurred at 35°C on a Metrosep Carb 2 (250/4.0) column behind a Metrosep Trap 1 100/4.0 guard column. The eluent was 20 mM NaOH at a flow rate of 0.8 mL min^{-1}. An IC amperometric detector (cycle: 300 ms 0.05V, 50 ms 0.55 V, 200 ms −0.1V detection during 200–300 ms of each cycle) was used for detection of eluted components. The sample aspiration needle was cleaned with acetone between each analysis. The lower limit of quantification was 79.2 mg L^{-1}. Standards and controls were regularly used for calibration of the signals.

Data analyses

A parametric fit of growth data for each replicate was made using non-linear least squares fitting with four different parametric models, i.e. the Gompertz, Exponential Gompertz, Richards and logistic growth curve models, using Grofit package v1.1.1-1 (Kahm *et al.*, 2010) in R 3.2.3. (data was ln(N/N0) transformed for specific growth rate fitting (Zwietering *et al.*, 1990). The model with the best Akaike Information Criterion is returned. Non-parametric fitting using lowess is used to perform initial parameter estimation. Generation time was calculated based on specific growth rate. For significance testing, one-way ANOVA with Tukey HSD contrasts was run if the data was homoscedastic (Modified Levene's test) and the residuals normally distributed (Shapiro-Wilks normality test). If the homoscedasticity assumption was not met, Weighted least squares was performed with Tukey simultaneous relative contrast effects for general linear hypotheses with a single-step method (Hothorn *et al.*, 2008), while if the normality assumption was not met non-parametric multiple contrast effects were used for the multiple comparison (Konietschke *et al.*, 2015).

Accession numbers

The Whole Genome Shotgun projects of *Methyloceanibacter* sp. R-67174, R-67175, R-67176 and R-67177 have been deposited at DDBJ/EMBL/GenBank under the accession numbers LPWG00000000, LPWF00000000, LPWE00000000 and LPWD00000000 respectively. The versions described in this paper are the first versions. The reads of the metagenomes of both enrichment cultures are available in the SRA database (study accession number SRP068992).

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Supporting information

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Fig. S1. Representatives of each *Methyloceanibacter* genotype based on $(GTG)_5$ (A) and Box (B) repetitive sequence based (Rep) PCR fingerprinting. Forty-one isolates assigned to the genus *Methyloceanibacter* based on partial 16S rRNA gene sequence similarity were grouped into four clusters, all distinct from *Methyloceanibacter caenitepidi* Gela4^T, with both techniques. Dendrograms were created using Pearson product moment correlation coefficient and UPGMA using BioNumerics 7.5.

Fig. S2. Phylogenetic Maximum Likelihood showing the affiliation of the novel *Methyloceanibacter* spp. based on the 16S rRNA gene. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [1] using 1322 nucleotide positions. The tree with the highest log likelihood (−9625.9027) is shown. Bootstrap values (1000 replicates) higher than 70 are shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.5937)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Alignment was performed using SILVA Incremental Aligner [2] and evolutionary analyses were conducted in MEGA6 [3].

Fig. S3. Physical map of genome environment of sMMO operon in R-67174, closely related and other

methanotrophs. Homologs are depicted in identical colors. ORF of hypothetical proteins are given as white arrows. ME, ORF for mobile element. Drawn to scale.

Fig. S4. Growth (◆) and methane oxidation (■) by R-67174 over time when both methane and methanol are provided as carbon source ($n = 6$). No methane was consumed and lag phase, specific growth rate and maximal cell density was not different from that on methanol alone (see Table 1). Growth on methanol (1%) or methane (5.4 mM) as sole carbon source are given for comparison as grey dotted and dashed lines respectively. All growth experiments were performed with addition of carbon dioxide.

Fig. S5. Phylogenetic Maximum Likelihood showing the affiliation of the methanol dehydrogenases. Representatives of MxaF, five Xox types, and nine clades of other type I and II alcohol dehydrogenase quinoproteins were chosen based on Keltjens *et al*. (2014) [4]. Sequences from this study are given in bold. For further explanation on subgrouping of XoxF1, see text.

Table S1. Genome statistics. Details on genome of Gela4^T are included for convenience of comparison. Number of CDS and RNA taken from RAST annotation.

Table S2. Genomic taxonomy of the *Methyloceanibacter* spp.

Table S3. Genome inventory associated with C1 and nitrogen metabolism.

Table S4. Differential growth responses (OD_{600}) of the *Methyloceanibacter* spp.